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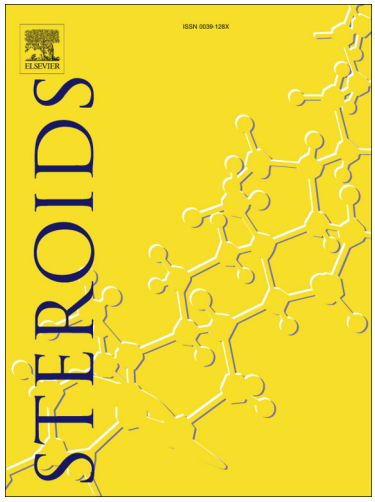

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Endogenous steroid hormones in hair: investigations on different hair types, pigmentation effects and correlation to nails

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Abstract: Steroid hormone analysis is widely used in health- and stress-related research to get insights into various diseases and the adaption to stress. Hair analysis has been used as a tool for the long-term monitoring of these steroid hormones. In this study, a liquid chromatography-tandem mass spectrometry method was developed and validated for the simultaneous identification and quantification of seven steroid hormones (cortisone, cortisol, 11-deoxycortisol, androstenedione, 11-deoxycorticosterone, testosterone, progesterone) in hair. Cortisol, cortisone, androstenedione, testosterone and progesterone were detected and quantified in authentic hair samples of different individuals. Significantly higher concentrations for body hair were found for cortisone and testosterone compared to scalp hair. Furthermore, missing correlations for the majority of steroids between scalp and body hair indicate that body hair is not really suitable as alternative when scalp hair is not available. The influence of hair pigmentation was analyzed by comparing pigmented to non-pigmented hair of grey-haired individuals. The results showed no differences for cortisol, cortisone, androstenedione, testosterone and progesterone concentrations ($p > 0.05$) implying that hair pigmentation has not a strong effect on steroid hormone concentrations. Correlations between hair and nail steroid levels were also studied. Higher concentrations of cortisol and cortisone in hair were found compared to nails ($p < 0.0001$). No significant correlation for cortisone, cortisol, androstenedione, testosterone and progesterone concentrations were found between hair and nails. These results demonstrate that matrix-dependent value ranges for hair and nail steroid levels should be established and applied for interpretation.

Keywords: endogenous steroids, liquid chromatography-tandem mass spectrometry, keratinized matrices, stress

Introduction

The hypothalamic-pituitary-adrenal (HPA) axis is regulated by cortisol and related steroid hormones [1]. The simultaneous analysis of steroid hormones is important for many clinical questions (e.g. adrenal insufficiency) and psychobiological inquiries (e.g. stress research) [2]. The most prominent and most studied steroid hormone is cortisol which is known as biomarker for stress [3]. The long-term determination of cortisol and other steroid hormones in the keratinized matrix hair has become an important tool in recent years [2]. While steroid concentrations in urine, blood and saliva underlie daily fluctuations, the hair matrix allows a retrospective measurement of accumulative concentrations over a longer period of up to several months [4]. In forensic toxicology as well as for clinical investigations, scalp hair is always the first choice for hair analysis due to the more accurate determination of the corresponding time frame via the growth rate. If no scalp hair is available due to medical or cosmetic reasons, it was shown that for forensic purposes, chest, arm and leg hair can be used as alternative for monitoring alcohol and drug consumption or abstinence [5]. No information about the applicability of body hair is available to date for steroid hormones. Two main factors influence the estimation of the time frame that corresponds to the hair length: the growth rate during the anagen phase and the percentage of hair in the telogen phase. These two factors differ significantly between body and scalp hair. The growth rate in the anagen phase for chest hair is approximately 0.8-1 cm/month, for arm hair 0.9 cm/month, for leg hair 0.4-0.7 cm/month and for scalp hair it can vary between 0.6 to 1.5 cm/month [5]. The

percentage of telogen hair is much higher in body hair (50-80%) compared to scalp hair (5-20%) [5]. Due to these varying growth rates and percentages of hair in the telogen phase, it is difficult to estimate a common time windows for body hair and the corresponding scalp hair [5].

Some key factors influence substance incorporation into hair including the melanin content of the hair and the lipophilicity and basicity of the substance itself. The effect of pigmentation (melanin-effect) on drug incorporation has been demonstrated for drug incorporation into grey hair [6]. Grey hair is a mixture of white and pigmented hair. Pigmented hair (black, brown) has a higher melanin content than less (blond, ginger) and non-pigmented (white) hair. Eumelanin is responsible for the pigmentation of dark hair whereas pheomelanin is more present in blond hair [7]. The interactions with pheomelanin are less important because they are typically weaker than those with eumelanin [8]. Despite hair root exposure to the same substance concentration in blood, the concentration of basic drugs in pigmented hair can be 10-fold higher than in non-pigmented hair [6]. No differences in drug incorporation are observed for neutral or acidic compounds [6, 9]. Furthermore, the pKa and the pH of matrix cells are important. The intracellular pH of keratinozytes and melanozytes has been found to be acidic which favors the distribution equilibrium of basic substances into the more acidic matrix cells. Both effects, the lower pH in matrix cells and the melanin binding lead to the accumulation of lipophilic basic substances into matrix cells with clear preference for pigmented cells. Therefore, acidic substances are only found in very low concentrations in hair [10].

Nails can be used as an alternative matrix for the assessment of steroid hormones, if neither scalp nor body hair is available [11-14]. Nails have an overall lower growth rate than hair. Nail growth rates differ significantly between hand and feet and even

between the fingers of one hand [1] which makes interpretation of nail results challenging. However, recent studies showed that steroid hormones can be detected in nails and are used as a tool in newborns to monitor *in utero* stress exposure [14, 15].

Determination of steroid hormones in keratinized matrices can be done with several analytical methods including immunoassays [16], GC-MS [17] and LC-MS [2, 18-20]. Immunoassays are often used because they can be performed easily and rapidly but cross-reactivity with other steroids can lead to an overestimation of steroid levels in hair and different assays are needed for multi steroid analysis [2]. For GC-MS, time-consuming derivatization steps and long run times are necessary, thus it is not feasible when a large number of samples needs to be processed. In recent years, LC-MS methods for the simultaneous determination of steroid hormones have become more and more important [2]. LC-MS/MS methods have higher sensitivity and specificity compared to other analytical methods and easier sample preparation.

In our study, an LC-MS/MS method for the simultaneous determination and quantification of seven steroid hormones in hair was developed and steroid levels in hair were systematically investigated. The goals of the study were firstly to investigate whether not only scalp hair but also body hair is suitable for steroid analysis. Furthermore, the influence of pigmentation on steroid concentration as well as correlations between steroid levels in hair and nails were investigated.

Experimental

Study design

The study was performed in full accordance with Swiss laws (statement of Cantonal Ethics Board of the Canton of Zurich: BASEC-Nr. Req-2017-00946). For validation, a homogenous pool of hair from healthy volunteers was prepared by milling the hair. Cohort A was used for the analysis of authentic hair samples and included 38 subjects. These subjects were recruited previously in the frame of a study to investigate the benefits of nature on health [21]. Corresponding hair and toenail samples were collected from the 38 individuals. Cohort B included 60 male subjects and was obtained from our routine case work of the Centre for Forensic Hair Analytics. A comparison of steroid levels was carried out from this cohort with corresponding scalp and body hair samples (leg hair n=28, arm hair n=20, chest hair n=12). Cohort C included 18 grey-haired subjects which were obtained from our routine case work as well as from healthy volunteers. Investigations of pigmentation on steroid levels were conducted from this cohort.

In general, hair samples were collected from the posterior vertex region and cut as close to the scalp as possible and cut into 3 cm segments. The 3 cm hair segment proximal to the scalp represents the hair growth over the last three month prior to the sampling, based on the average growth rate of 1 cm/month. Secondary hair from chest, arms and legs was shaved off with a disposable razor. Toenails were sampled from the big toe. All samples were washed for 3 min with 15 mL of deionized water, followed by washing for 2 min with 10 mL acetone. The washing solutions were decanted and disposed. The samples were dried overnight at room temperature.

Chemical reagents

Cortisone, cortisol, 11-deoxycortisol, androstenedione, 11-deoxycorticosterone, testosterone, and progesterone (all 1 mg/mL) were purchased from Sigma Aldrich (Buchs, Switzerland) as well as deuterated internal standards (cortisone-D_7,

progesterone-D_9, all 0.1 mg/mL). $^{13}C_3$-cortisol and $^{13}C_3$-cortisone (both 0.1 mg/mL) were purchased from Isoscience (USA) and $^{13}C_3$-androstenedione, $^{13}C_3$-progesterone and $^{13}C_3$-testosterone (all 0.1 mg/mL) from Sigma Aldrich (Buchs, Switzerland). Water and methanol were of LC–MS grade (Chromasolv®) and purchased from Sigma-Aldrich (Buchs, Switzerland). Acetone, ethyl acetate and ammonium fluoride were purchased from Merck (Darmstadt, Germany). Reconstitution solution consisted of 0.2 mM NH_4F in water/methanol 97/3 v/v. All chemicals were of highest analytical grade available.

Preparation of standard stock solutions

Standards of each steroid hormone were prepared in methanol with final concentrations of 1 ng/µL. The solutions were further individually diluted with methanol to give a working standard solution for each steroid hormone. The internal standard mixture (cortisone-D_7, progesterone-D_9) was prepared to a final concentration of 40 pg/µL. All stock solutions were stored at −20°C until use.

LC-MS/MS Analysis

A liquid chromatography-tandem mass spectrometry (LC-MS/MS) device was used for the analysis of the samples. Analytes were separated with a Prominence UFLC system (Shimadzu, Kyoto, Japan) by injecting 10 µL of the samples onto a Phenomenex® Kinetex® XB-C_{18} (2.6 µm, 50 x 2.10 mm) column. The mobile phase consisted of 0.2 mM NH_4F in water/methanol 97/3 v/v (A) and 0.2 mM NH_4F in water/methanol 3/97 v/v (B). The flow rate was 0.45 mL/min and the temperature of the column oven was set to 40 °C. Separation was achieved by the following gradient: 0–40% B for 0–0.1 min, 40–50% B from 0.1–5 min, isocratic 50% from 5 to

8 min, 50–90% B from 8–11 min, isocratic 90% B from 11 to 14 min, 90–40% B from 14 to 15 min followed by an equilibration step of 1 min. Detection was performed with a QTRAP® 6500+ linear ion trap quadrupole mass spectrometer (Sciex, Darmstadt, Germany) equipped with an electrospray ionization (ESI) source. Multiple reaction monitoring mode (MRM) with an ion spray voltage of 5500 V was used to for quantification. Steroid hormones were measured in positive electrospray ionization mode. The parameters of the optimized method used for validation and the analysis of the authentic samples are listed in Table S1. The curtain gas was fixed at 20 psi, the collision gas was set to medium, the ion source gas 1 at 70 psi and ion source gas 2 set at 50 psi and the source temperature was set to 450 °C. Analyst® software (version 1.6.3, Sciex, Darmstadt, Germany) was used for instrument control and data analysis.

Extraction

For extraction, 20 mg hair was weighed in an Eppendorf tube and milled for 10 min at a frequency of 30 Hz. Then 50 µL IS (40 pg/µL cortisone-D_7, progesterone-D_9) and 1 mL methanol were added. The samples were briefly shaken and placed in a sonication bath for 2 hours at 55 °C for extraction. After centrifugation for 5 min at 9000 g, the methanolic extract was transferred into pill glasses and dried under nitrogen at 35 °C. A liquid-liquid extraction (LLE) was carried out by adding 1.5 mL ethyl acetate and 220 µL of water to the dried extract. The resulting mixture was subsequently shaken for 10 min at 5 Hz. The samples were centrifuged for 5 min at 9000 g and then placed in a freezer (-20 °C) for approximately 60 min. The upper ethyl acetate layer was poured off and dried under nitrogen at 35 °C. All samples were reconstituted in 150 µL MeOH and 350 µL reconstitution solution and

centrifuged (5 min, 9000 g) in centrifugal filters before transferring them into LC-MS/MS vials.

Validation in hair

Validation was performed in accordance with the guidelines of the GTFCh appendix C for hair [22]. If available, $^{13}C_3$-labeled steroid hormones ($^{13}C_3$-cortisone, $^{13}C_3$-cortisol, $^{13}C_3$-androstenedione, $^{13}C_3$-testosterone, and $^{13}C_3$-progesterone) were used as surrogate analytes as previously described [15]. For the two other analytes (11-deoxycortisol, 11-deoxycorticosterone,) isotope-labeled compounds were not commercially available and authentic hair matrix which was sufficiently low in concentration was used as blank matrix and spiked. The following parameters were tested: response factor (only for the surrogate analytes), linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision, matrix effects, recovery and robustness. The response factor was calculated by the ratio of the peak areas found for surrogate and unlabeled analyte. If the response factor was not 1, it was incorporated into the regression line of the calibration curve [15].

The limit of detection (LOD) and limit of quantification (LOQ) was determined by the lowest amount of spiked standards in the hair matrix that still provided a signal-to-noise ratio of 3:1 and of 10:1, respectively. For linearity, six calibrators with increasing concentrations were prepared (s. Table S2).

For accuracy and precision, duplicates of a hair pool spiked with different concentrations according to the various substance classes were prepared (s. Table S3). For the $^{13}C_3$-labeled analytes three quality controls were prepared with different end concentrations. For $^{13}C_3$-cortisone, $^{13}C_3$-cortisol, $^{13}C_3$-androstenedione, $^{13}C_3$-testosterone and $^{13}C_3$-progesterone, end concentrations were 1.2 pg/mg (QC low),

40 pg/mg (QC medium) and 400 pg/mg (QC high). For 11-deoxycortisol and 11-deoxycorticosterone, end concentrations were 3 pg/mg (QC low), 40 pg/mg (QC medium) and 400 pg/mg (QC high). The measurements were carried out on six consecutive days. The bias as well as the intra- and inter-day precision were calculated for each analyte in each matrix (s. Table S3).

For matrix effect and recovery, five replicates of hair samples from different individuals with low and high concentration levels (Table S3) were analyzed. For matrix effect, the ratio of peak areas of spiked hair (A) to spiked solvent (B) at the same concentration was compared (Matrix effect = (A/B) x 100). For recovery, the ratio of peak area of spiked hair before extraction (C) to spiked hair after extraction (D) was compared (Recovery = (C/D) x 100).

For robustness, six replicates of an authentic hair pool were analyzed and the mean value of detectable hormones and the relative standard deviation was determined. In addition, a hair pool was analyzed using different sample weights (1 mg, 2 mg, 4 mg, 7 mg, 10 mg, and 20 mg, respectively) to evaluate whether the sample amount has an influence on the measured concentration.

Measurement in nails

Nails were analyzed as described in Voegel et al. [15].

Statistics

Statistical analysis was conducted with GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA). Shapiro Wilk normality test revealed that all data were not normally distributed. Paired samples were compared using Wilcoxon signed-rank test and unpaired samples were compared using Mann-Whitney test. Correlation

coefficients were calculated using Spearman correlation. *P*-values> 0.05 were considered as not statistically significant (ns); $p< 0.05$ (*) as significant; $p< 0.01$ (**) as very significant; $p< 0.001$ (***) and $p< 0.0001$ (****) as extremely significant.

Results and Discussion

Analytical method and validation

As recently published, we developed a sensitive and selective LC-MS/MS method for the quantification of seven steroid hormones in human nails [15]. In this study, the method was adapted for the analysis of steroid hormones in hair. It could be shown that also in the hair matrix seven analytes could be detected with within a total run time of 12 minutes. A methanolic extraction combined with an LLE for sample clean-up enabled a rapid throughput of hair and nail samples.The calibration curves (Table S2) showed good linearity for all analytes (correlation coefficient > 0.99). The response factor was calculated and applied for each surrogate analyte and the corresponding authentic analyte (Table S3). The principal of surrogate analytes was described in our previous publications [15, 23] . The LOQ values were 0.3 pg/mg for $^{13}C_3$-testosterone, 0.5 p/mg for 11-deoxycortisol, $^{13}C_3$-androstenedione and $^{13}C_3$-progesterone, 1 pg/mg for $^{13}C_3$-cortisone and $^{13}C_3$-cortisol and 5 pg/mg for 11-deoxycorticosterone.

Results for accuracy are summarized in Table S4. Accuracy was good for all analytes and was in the accepted range of ± 25%. Intraday precision (RSD_r) ranged from 2.3 to 19.1%. Interday precision (RSD_T) results ranged from 2.7 to 24.9%.

Recovery was in an acceptable range above 71 % for all analytes as well as for the surrogate analytes (Table S4). Ion suppression was observed for all analytes in a

range of 72-85% which is still acceptable. Consequently, all validation parameters were fulfilled for the surrogate and non-labeled analytes.

For robustness, six replicates of a homogenized authentic hair pool were measured. Cortisol, cortisone, testosterone, progesterone and androstenedione could be detected in this authentic pool. The mean and the relative standard deviation were in an acceptable range below 20 % (cortisol 17.2 pg/mg ± 4.4 %, cortisone 87.1 pg/mg ± 7.4 %, testosterone 1.0 pg/mg ± 10.7 %, progesterone 13.7 pg/mg ± 13.1 %, androstenedione 1.0 pg/mg ± 7.1 %). The concentrations of cortisol, cortisone and progesterone measured using increasing sample weight showed consistent results with deviations below 5 % (cortisol 11.0 pg/mg ± 3.5 %, cortisone 83.0 pg/mg ± 4.5 %, progesterone 13.3 pg/mg ± 4.8 %). These results proofed that even samples with only small weight can be measured accurately. This is particularly important for hair analysis because it is not always possible to obtain a large amount of sample material.

Analysis of authentic hair samples

LC-MS/MS analysis was performed on 38 authentic hair samples from adults (25 female/13 male, mean age: 41 years, age range: 27-65 years) from cohort A. The mean concentrations determined were 23.4 pg/mg for cortisone, 4.9 pg/mg for cortisol, 2.4 pg/mg for androstenedione, 1.1 pg/mg for testosterone, and 5.0 pg/mg for progesterone (Table 1). Cortisol and cortisone measurements in hair are well established and values were similar to previously published ones [20, 24, 25]. Testosterone showed values of 0.3–2.7 pg/mg which is in good agreement with the concentrations determined in literature [26]. Further, androstenedione and

progesterone concentrations were in good accordance with previously published concentrations [24].

Steroid levels in in hair from male and female participants were conducted. Mann-Whitney test revealed that male participants had significantly higher cortisone ($p< 0.05$) and cortisol ($p< 0.01$) values than female participants. Progesterone concentrations showed a trend to be higher in females whereas testosterone and androstenedione values tended to be higher in males, but statistically no significant difference was found.

Previous studies found that hair cortisol was higher in male individuals [4, 26, 27]. It was hypothesized that men have lower corticosteroid binding globulin (CBG) levels and this might result in higher free cortisol levels [4]. Our results indicate that gender has an influence on hair steroid concentrations of cortisone, androstenedione, testosterone and progesterone. Reasons for that might be that blood testosterone levels are generally higher in male than in female individuals [24]. The higher hair progesterone levels in female individuals can be explained by the primarily secretion of progesterone from the ovaries [28].

The ratio of cortisol-to-cortisone was calculated to study the steroid metabolism. This ratio is an indirect measurement of the 11β-hydroxysteroid dehydrogenase activity which is the key enzyme in the intracellular conversion of cortisol in cortisone and vice-versa [29]. The mean ratio observed was 0.21 which is in line with previous research in hair [29]. If the ratio is less than 1, it indicates an increased conversion of cortisol to cortisone [19]. The ratio measurement is used in stress related research because 11β-hydroxysteroid dehydrogenase enzyme activity is altered under stress [30].

Table 1: Steroid hormone concentrations in pg/mg in 38 hair samples. F: female, M: male.

Sex	Age	Cortisone	Cortisol	Androstenedione	Testosterone	Progesterone
F	40	12.8	2.1	1.8	<LOQ	5.0
F	48	7.8	2.6	1.3	<LOQ	2.8
F	27	37.1	11.1	<LOQ	<LOQ	1.1
M	43	36.6	4.3	4.3	2.7	4.4
M	58	107.0	17.4	4.6	1.9	<LOQ
F	41	10.1	3.0	2.7	<LOQ	4.9
F	39	11.9	1.9	<LOQ	<LOQ	<LOQ
F	60	13.2	1.3	<LOQ	<LOQ	2.2
F	32	11.2	1.7	<LOQ	<LOQ	<LOQ
F	28	49.7	10.5	2.0	<LOQ	1.7
M	31	23.4	4.5	2.2	0.6	<LOQ
M	33	18.5	3.4	4.0	1.3	<LOQ
M	49	18.4	3.2	1.7	0.9	<LOQ
F	65	17.4	2.2	<LOQ	<LOQ	<LOQ
F	29	14.9	6.5	<LOQ	<LOQ	2.5
M	43	18.2	4.4	2.7	1.4	2.4
F	56	9.4	1.5	<LOQ	0.3	2.5
F	51	16.1	4.0	3.0	<LOQ	3.9
M	30	12.1	4.7	1.8	0.8	<LOQ
F	35	10.5	2.3	<LOQ	<LOQ	3.0
F	61	14.7	2.2	<LOQ	<LOQ	<LOQ
F	31	12.8	2.3	<LOQ	<LOQ	6.1
M	47	133.0	36.1	2.8	0.8	1.0
F	39	17.9	4.3	<LOQ	<LOQ	4.6
F	30	17.1	2.9	<LOQ	<LOQ	<LOQ
F	39	18.2	2.8	<LOQ	<LOQ	1.6
M	34	10.9	2.5	2.0	0.7	<LOQ
F	65	16.5	2.3	<LOQ	<LOQ	<LOQ
F	39	14.3	3.3	1.0	<LOQ	<LOQ
F	31	14.1	3.0	2.0	<LOQ	3.8
F	44	15.8	3.3	1.7	<LOQ	2.1
M	45	16.5	2.9	2.4	0.8	0.9
F	31	14.3	2.1	1.9	<LOQ	5.4
M	31	33.5	6.4	<LOQ	0.6	<LOQ
M	39	8.1	2.2	<LOQ	<LOQ	1.5
M	48	33.5	6.4	2.7	1.6	2.8
F	37	21.8	3.9	3.3	<LOQ	47.8
F	27	19.4	3.2	1.9	<LOQ	<LOQ
Mean:	41	23.4	4.9	2.4	1.1	5.0
Median:	39	16.3	3.1	2.1	0.8	2.8
Min:	27	7.8	1.3	1.0	0.3	0.9
Max:	65	133.0	36.1	4.6	2.7	47.8

Steroid levels in scalp and body hair

Scalp and the corresponding body hair samples (leg n=28, arm n=20, chest n=12) were obtained from 60 male subjects from cohort B. Intra-individual steroid levels were compared in scalp and body hair (see Figure 1). Testosterone levels ($p < 0.0001$) and cortisone levels ($p < 0.05$) were significantly higher in body hair than in scalp hair) (see Figure 1). For cortisol, androstenedione and progesterone no significant difference between body and scalp hair (Wilcoxon signed-rank test, $p > 0.05$) could be observed.. One explanation for this effect could be the habit of hair washing. It is known that washing can reduce steroid levels in hair, thus different habits in washing scalp hair and body hair can result in different concentration levels [27]. Increased incorporation of compounds via sweat into body hair is another possibility for higher concentrations in body hair. Spearman correlation coefficients between scalp and body hair were found not to be significant for cortisol ($r=0.13$, $p>0.05$) whereas cortisone ($r=0.42$, $p<0.001$), androstenedione ($r=0.63$, $p<0.0001$), testosterone ($r=0.50$, $p<0.0001$), and progesterone ($r=0.37$, $p<0.05$) showed a correlation. When comparing the different body hair types it became obvious that mostly chest hair showed a good correlation with scalp hair for the majority of steroid hormones. Leg hair showed correlations for androstenedione and testosterone. Arm hair only showed a correlation for progesterone, but the number of pairs was only four, so that no real conclusion can be drawn. A reason for the missing correlation for arm hair might be the low sample amount that is normally achieved when using arm hair. Taken together the results show that steroids can be measured in body hair and indicate that chest hair might be the best sample type when using body hair.

Table 2: Spearman correlation coefficient for scalp and body hair.

Steroid Hormone	**Scalp vs. body hair**	**Scalp vs. leg hair**	**Scalp vs. chest hair**	**Scalp vs. arm hair**
Cortisone	0.42, $p < 0.001$	0.30, $p > 0.05$	0.69, $p < 0.001$	0.30, $p > 0.05$

Cortisol	0.13, p > 0.05	0.31, p > 0.05	0.03, p > 0.05	0.17, p > 0.05
Androstenedione	0.63, p < 0.0001	0.80, p < 0.0001	0.71, p < 0.001	0.12, p > 0.05
Testosterone	0.50, p < 0.0001	0.48, p < 0.01	0.49, p < 0.05	0.54, p < 0.05
Progesterone	0.37, p < 0.05	0.28, p > 0.05	0.48, p < 0.05	0.80 p > 0.05

Nevertheless significant differences in body hair for two steroids and missing correlations indicate that body hair is not really suitable as alternative when scalp hair is not available. Therefore, we strongly recommend using exclusively either scalp or body hair in one study cohort. A drawback for body hair is certainly the more uncertain time window which makes a retrospective interpretation more difficult. This should be taken into account when using body hair as sample type for any studies.

Influence of pigmentation

The influence of hair pigmentation was studied with grizzled hair from 18 individuals (6 female/12 male) from cohort C by subdividing the hair sample into pigmented and non-pigmented (white) hair. Pigmented and white hair of each individual was extracted and analyzed separately. No significant difference could be found between cortisol, cortisone, androstenedione, testosterone and progesterone concentrations ($p > 0.05$) in pigmented and white hair. Previous studies found contradictory effects of hair color on cortisol levels. First studies reported that cortisol concentration in hair is not affected by hair color [31] whereas later studies showed that black hair has higher cortisol levels than blond hair [4, 27]. It is hypothesized that cortisol binds to melanin through weak interactions like hydrogen bridge bonding or van der Waals forces. Considering our findings, the theory can be strengthened that rather weak interactions with melanin are responsible for the bonding of steroid hormones to the hair matrix than strong ionic bonding. Besides the binding to melanin, other biochemical processes within the hair root may be responsible for the steroid deposition. Since steroid hormones were found in non-pigmented hair, other binding

sites, like hair proteins or lipids, could be responsible for steroid binding in hair [6, 32]. The present study is the first one to describe the intra-individual comparison of five steroid hormones concentrations, thus eliminating factors of individual differences in metabolism. Since intra-individual comparisons of steroid hormones were studied, no conclusion on steroid levels in different hair colors could be drawn. Further investigations on steroids in bigger cohorts with different hair colors could confirm the presented theory.

Correlation of hair and nail samples

A total number of 38 hair and corresponding nail samples from cohort A were measured to determine steroid concentrations. Cortisol, cortisone, androstenedione, testosterone and progesterone were detected in hair and nails. Statistical analysis revealed higher concentrations of cortisol ($p< 0.0001$) and cortisone ($p< 0.0001$) in hair compared to nails, which confirmed results from previous studies (Figure 2) [1, 13]. No significant difference was found for androstenedione whereas testosterone ($p< 0. 05$) and progesterone ($p< 0. 05$) values were significantly higher in nails than in hair (Figure 2). No significant correlation for cortisone (r=0.07), cortisol (r=0.12), androstenedione (r=-0.05), testosterone (r=-0.20) and progesterone (r=0.37) concentrations were found between hair and nails. Several aspects influence the concentration levels in hair and nails such as growth rate, incorporation route, washing and keratin binding.

Hair and nails are mainly built up by keratin whereas melanin is responsible for the pigmentation in hair and is not present in nails. Since nails do not contain melanin, the incorporation of substances is either reduced or enhanced depending on the lipophilicity [33]. For example, cortisol and cortisone are rather lipophilic, showing lower concentrations in nails [1] compared to ethyl glucuronide which is hydrophilic

[34]. As testosterone ($p< 0.05$) and progesterone ($p< 0.05$) showed a trend for higher values in nails this further strengthens the hypothesis that steroid hormones bind to melanin only through weak interactions. Other factors than melanin binding could be responsible for the incorporation into the two different matrices or other binding sites than melanin might play a role as discussed above.

Furthermore, influences like washout effects in pre-analytical treatment should be taken into account. Since hair and nails do have different structure surfaces, pre-analytical washing can possibly wash out substances to different extents and thus influences their concentrations in the matrices.

To the best of our knowledge, this study investigated for the first time different steroid hormones in the hair and nail matrix. We could show that both matrices are suitable for steroid analysis but they should not be used as alternative samples in the same study cohort. Further studies with larger sample cohorts have to be conducted to confirm the observed differences.

Conclusion

An LC-MS/MS method for the determination and quantification of seven steroid hormones in hair was developed and validated. The method allowed us to investigate steroid levels in scalp hair compared to body hair. The results indicated that chest, arm and leg hair cannot be used as an alternative sample types when no scalp hair is available. Further investigations in pigmented and non-pigmented hair showed that steroid hormones are rather bound through weak interactions to melanin and other binding sites might play a role too. No correlations between hair and nails indicated that only one matrix should be chosen for study cohorts and

matrix-dependent value ranges have to be applied. The data presented should help to further establish steroid measurements in hair and nails.

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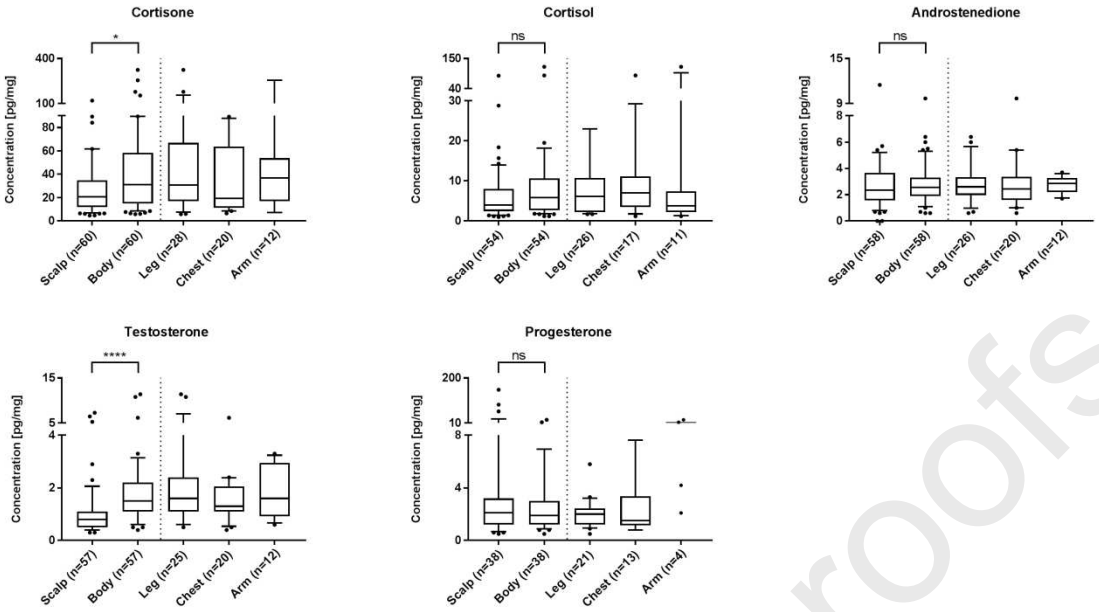
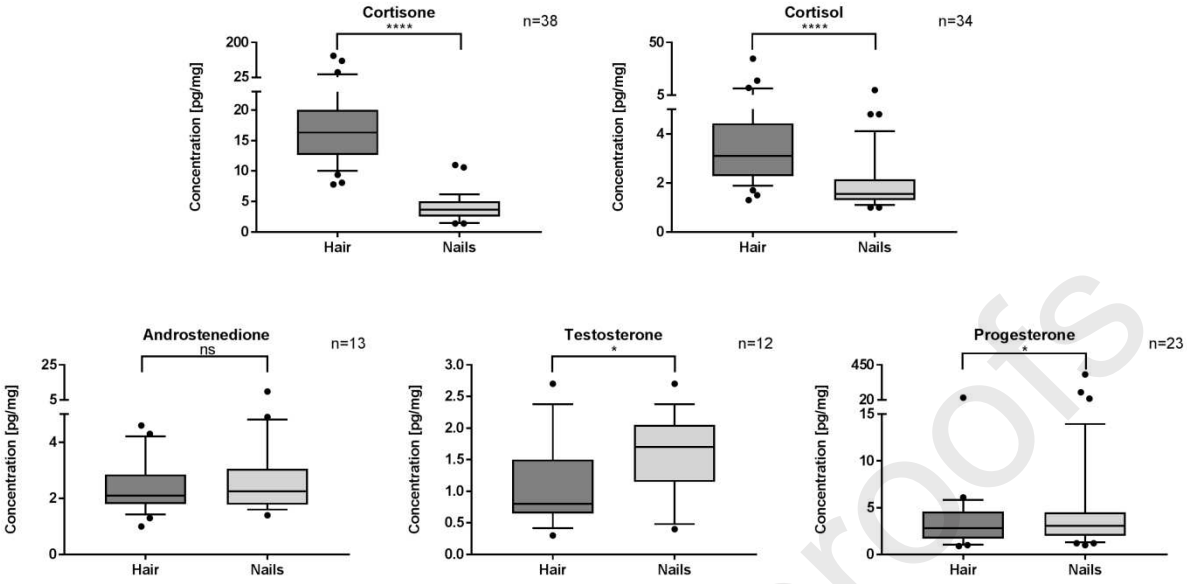
Figure 1: Comparative statistical analysis of steroid concentrations compared in scalp and body hair and representative box plots of leg, chest and arm hair. The black line in middle represents the median. The boxes represent the 25% and 75% percentile. The whiskers represent the 10% and 90% percentile. Statistical analysis was done by Wilcoxon-signed rank test. Significance level is indicated with asterisks. *P*-values> 0.05 were considered as not significant (ns), $p< 0.05$ (*) as significant, $p< 0.0001$ (****) as extremely significant. n=number of pairs. Only pairs with values over LOD were considered for statistical analysis.

Figure 2: Comparative statistical analysis of steroid concentrations compared in hair and in nails. The black line in middle represents the median. The boxes represent the 25% and 75% percentile. The whiskers represent the 10% and 90% percentile. Statistical analysis was done by Wilcoxon-signed rank test. Significance level is indicated with asterisks. *P*-values> 0.05 were considered as not significant (ns), $p< 0.05$ (*) as significant, $p< 0.0001$ (****) as extremely significant. n_p=number of pairs.





Highlights

- An LC-MS/MS method was developed and validated for the quantification of 12 seven steroid hormones in hair
- 5 Five steroids were detected in authentic hair samples
- No significant difference Differences for most steroids was were found between body and scalp hair
- Pigmentation had no strong influence on steroid concentrations
- No correlations were found between hair and nail steroid levels